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Ministry of Economic Development
Manatū Ōhanga
Intellectual Property Office of New Zealand

PCT/NZ2004/000323

CERTIFICATE

This certificate is issued in support of an application for Patent registration in a country outside New Zealand pursuant to the Patents Act 1953 and the Regulations thereunder.

I hereby certify that annexed is a true copy of the Provisional Specification as filed on 19 December 2003 with an application for Letters Patent number 530296 made by KERATEC LIMITED.

Dated 11 January 2005.

Neville Harris
Commissioner of Patents, Trade Marks and Designs

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Patents Act 1953

PROVISIONAL SPECIFICATION

WOUND CARE PRODUCTS CONTAINING KERATIN

We, **KERATEC LIMITED,** a New Zealand company, of Springs Road, Lincoln, Canterbury, New Zealand do hereby declare this invention to be described in the following statement:

PT043888530

WOUND CARE PRODUCTS CONTAINING KERATIN

Field

The invention relates to wound care products containing keratin.

Background of the invention

Wounds and lesions can be caused by a variety of events, including surgery, traumatic injury, burns, abrasions and skin grafts. Healing of wounds may be difficult and may result in problems such as ulcers and septicemia. Of particular concern are chronic wounds, such as pressure sores and diabetic ulcers. The treatment of these conditions is of increasing importance as the population ages. The conventional cascade of biochemical processes which occurs in wound healing, involving hemostasis and inflammation, granulation tissue formation and reepithelization and remodeling, is disrupted in the case of chronic wounds due in part to the prolonged inflammatory response which occurs, and the release of destructive enzymes by inflammatory cells.

It has been recognized for some time that maintaining a moist environment can improve the rate of wound healing. Many products have been developed which provide this environment in order to increase the rate of repair of chronic wounds. The materials used in these dressings are biocompatible to some extent, and include polylactic acid, chitin, alginate derivatives and collagen. The response of these materials to wound exudates, and the biochemical environment that these materials provide are fundamental to their performance in the wound.

Keratin proteins are present in a wide range of biological tissue, performing a structural role in skin, hair and other materials. Keratins extracted from hair have been shown to be a valuable component in wound dressings. US 5932552 provides a biocompatible keratin material prepared by either reduction or oxidation for use as a component in wound care products. Those methods included in the art for the oxidation of keratins to create a polar group are harsh and degrading to the keratin, causing protein damage and loss of core physical characteristics arising from the protein amino acid composition and tertiary structure. In addition the oxidation processes used in the preparation of these materials are irreversible and the cysteic acid groups formed cannot be reconverted to cystine to perform a useful structural function. Those methods included in the art for reduction to

create soluble proteins are conducted under harsh alkaline conditions that also cause damage to the protein and loss of the core physical characteristics of the keratin proteins.

The core components of keratin fibres, especifically the intermediate filament proteins and the matrix proteins present in wool and hair, play particular roles within the fibre which is reflected in their tertiary structure and amino acid composition. These same features can be capitalized upon to create materials with good physical properties and highly absorbing capacities when using purified forms of these proteins. In order to do this, methods used for isolating keratins need to be mild, to prevent protein damage, create cystine modifications that are reversible, to allow for reconstitution of tough materials through the creation of cystine bonds, and facilitate the isolation of specific keratin protein fractions from the keratin source. The present invention provides new materials for use in wound care products that are prepared according to these principles.

Object of the Invention

It is an object of the invention to provide a wound care product which uses an intact keratin protein fraction. It is a further object of the invention to provide a keratin protein fraction that is S-sulfonated for use in wound care, or to at least provide the public with a useful choice.

Summary of the Invention

The invention provides a wound care product including an intact keratin protein fraction.

Further, the invention provides a wound care product including an S-sulfonated keratin protein fraction.

The keratin protein fraction may be S-sulfonated and intact.

The keratin protein fraction may be from the keratin intermediate filament protein family, or from the keratin high sulfur protein family.

The invention also provides the use of an S-sulfo keratin protein fraction formed as a freeze dried sponge in wound care products.

The invention also provides the use of S-sulfo keratin protein fractions as a spray or freeze dried powder in wound care products.

Also provided is the use of S-sulfo keratin protein fractions formed as a film in wound care products.

Also provided is the use of S-sulfo keratin protein fractions formed as a fibre in wound care products.

Also provided is the use of an intact keratin protein fraction formed as a freeze dried sponge, a spray or freeze dried powder, a film and a fibre in wound care products.

The fibre may be formed into a woven or non-woven matrix.

Materials combining S-sulfonated keratin protein fractions as wound care products are also provided.

The invention also provides a wound care product selected from the group comprising:

a freeze dried sponge;
a spray or freeze dried powder;
a film;
a fibre;
a sheet;
a dressing;
a bandage; and
a plaster;
and including an intact and/or S-sulfonated keratin product.

Any of the materials described above may be treated with reductants, which remove the s-sulfo function and reform cystine.

Any of the above materials may be used as a vehicle for the delivery of bioactive agents to a wound.

The keratin may be derived from any suitable source such as hair, wool, feathers, fur, nails, horns, hooves, claws, beaks or the like.

The invention also provides a method of treating a wound, lesion, burn, pressure sore, ulcer or the like comprising applying an intact s-sulphoned keratin product to the wound, lesion, burn, pressure sore, ulcer or the like site.

The invention also provides a method of creating a wound dressing comprising:

An S-sulfonated keratin material (such as a sponge, powder or fibre construct) in contact with the wound site, and another material, such as a film derived from S-sulfonated keratin or another material, as a backing to the dressing.

An S-sulfonated keratin material (such as a sponge, powder or fibre construct) containing bioactive agents in contact with the wound site, and another material, such as a film derived from S-sulfonated keratin or another material, as a backing to the dressing.

An S-sulfonated keratin material (such as a sponge, powder or fibre construct) not in contact with the wound site, but contained within a porous construct made from conventional wound care products, or from keratin.

Brief Description of the Drawings

The invention will now be described, by way of example only, with reference to the following specific embodiments:

Figure 1: shows sheep fibroblast proliferation on biopolymers;

Figure 2: shows human fibroblast proliferation on biopolymers;

Figure 3: shows the effect of Con(A) stimulation on T-cell growth in the presence of keratin matrices; and

Figure 4: shows the effect of Con(A) on T-cells.

Detailed Description of the Invention

The hard alpha keratin proteins such as those derived from human hair, wool, animal fibres, horns, hooves or other mammalian sources, can be classified into particular components according to their biochemical properties, specifically their molecular weight

and amino acid composition. Table 1 illustrates the amino acid composition determined by conventional analytical methods of typical keratin protein fractions known in the art and also the subject of this invention. This involves acid hydrolysis of the analyte which converts all cystine and labile cystine derivatives to cysteine, typically recorded as half-cystine.

	SIFP	SHSP	SPEP	IFP	HSP	Whole wool
Cya	0.4	1.7	0.7	0	0	0
Asp	7.9	2.6	8	9.6	2.3	5.9
Glu	15.4	8.6	15	16.9	7.9	11.1
Ser	10.9	14.3	11.4	8.1	13.2	10.8
Gly	8.1	9.1	8.4	5.2	6.2	8.6
His	0.9	0.8	0.9	0.6	0.7	0.8
Arg	7.9	6.8	6.9	7.9	6.2	6.2
Thr	6.5	10.4	6.5	4.8	10.2	6.5
Ala	7.5	3.6	7.5	7.7	2.9	5.2
Pro	5.4	12.6	5.7	3.3	12.6	6.6
Tyr	1.1	1.8	1.2	2.7	2.1	3.8
Val	6.5	6.3	5.8	6.4	5.3	5.7
Met	0.2	0	0.3	0.6	0	0.5
Lan	0.2	0.2	0.3	0	0	0
Ile	3.7	2.9	3.4	3.8	2.6	3
Leu	8.9	3.9	8	10.2	3.4	7.2
Phe	2.5	1.5	2.1	2	1.6	2.5
Lys	2.1	0.4	2.1	4.1	0.6	2.7
Cys	4.2	12.4	4.6	6	22.1	13.1

Table 1: amino acid composition of keratin fractions: S-sulfonated keratin intermediate filament protein (SIFP), S-sulfonated keratin high sulfur protein (SHSP), S-sulfonated keratin peptide (SPEP) as used in the invention. Intermediate filament protein (IFP), high sulfur protein (HSP) and whole wool courtesy of *Gillespie and Marshall, Variability in the proteins of wool and hair, Proc. Sixth Int. Wool Text. Res. Conf., Pretoria, 2, 67-77, 1980.* All residues expressed as mol%. S-sulfocysteine, cystine and cysteine are measured as S-carboxymethyl cysteine following reduction and alkylation, and reported as cys.

Table 2 illustrates the molecular weight determined by conventional analytical methods of typical keratin protein fractions known in the art and also the subject of this invention. Conventional analysis involves cleavage of cystine bonds within the keratin using reduction so that the protein mass is determined in its native, uncrosslinked state, most similar to the unkeratinsed state of the protein. Mass is determined using polyacrylamide gel electrophoresis. In the case of the peptide SPEP mass is determined using mass

spectrometry. Using these methods the keratin is made soluble without any hydrolysis of peptide bonds and an accurate measure of molecular weight is determined.

Keratin protein fraction	Molecular weight/kD
SIFP	45-60
SHSP	10-25
SPEP	<1
IFP	45-60
HSP	11-23

Table 2: Molecular weight of keratin fractions: S-sulfonated keratin intermediate filament protein (SIFP), S-sulfonated keratin high sulfur protein (SHSP), S-sulfonated keratin peptide (SPEP) as used in the invention. Intermediate filament protein (IFP) and high sulfur protein (HSP) courtesy of *Gillespie and Marshall, Variability in the proteins of wool and hair, Proc. Sixth Int. Wool Text. Res. Conf., Pretoria, 2, 67-77, 1980.*

Both amino acid composition and molecular weight varies across keratin types, between species and also within breeds of one species, for example between wools from different breeds of sheep. The figures given in Tables 1 and 2 are indicative for the keratin source stated. However, individual types of keratin proteins, or keratin protein fractions, have distinctive characteristics, particularly molecular weight and amino acid content.

Highly S-sulfonated keratins have been shown to be able to be formed into a variety of matrices including porous sponges, films and fibres using methods such as those outlined in NZ/PCT/00169 (which is incorporated herein).

Purified wool keratin intermediate filament proteins are particularly well suited to reformation into matrices, due in part to their high molecular weight and their tertiary structure. Methods outlined in NZ/PCT/00169 make extensive use of these materials to form useful matrices.

S-sulfo keratins can be prepared by a variety of methods, including those outlined in PCT/NZ02/00125 (which is incorporated herein).

Porous sponge matrices are of particular use in a wound environment as they can play an important role in absorbing wound exudates and maintaining a healthy environment for healing a wound. In addition they can act as media for the delivery of other healing agents, such as growth factors, antibacterial agents or cultured cells, that stimulate the healing process. These features are enhanced through using S-sulfonated keratin protein fractions to construct the matrices. The highly polar nature of the S-sulfo group makes matrices derived from this material highly absorbing. In addition, S-sulfonated keratins are biocompatible and do not invoke an adverse response in vitro.

Films are an important component in dressings for the treatment of wounds, providing a barrier to protect the wound and maintaining an appropriate environment to encourage healing. S-sulfonated keratins films are biocompatible and do not invoke an adverse response in vitro. As such, they are useful components in a wound dressing.

Fibres reconstituted from S-sulfonated keratin intermediate filament proteins can be used as a component for wound dressings. Fibres are particularly versatile as they can be formed into woven or non-woven constructs and fibre design can be used as well control of the chemistry of the material to effect the interaction of the dressing with the wound. Much work has been undertaken into the use of regeneratred fibres in wound care, in particular alginate fibres. Reconstituted keratin fibres derived from S-sulfonated intermediate filament proteins are a new material for use in similar applications.

Keratin materials derived from the SIFP and SHSP protein fractions contain differing amounts of the highly polar S-sulfo group, and consequently differ in their physicochemical characteristics, in particular their ability to absorb moisture. Wound dressings derived from a combination of these absorb moisture to a greater or lesser extent, and so can be controlled in the degree to which they will absorb wound exudates.

S-sulfonated keratin proteins prepared as spray or freeze dried powders are highly absorbing materials that are a valuable component in wound dressings, in particular for use in the hydrogel type dressing in which alginates or collagen derivatives are the materials used frequently in currently available products. Combination of the SIFP and SHSP proteins leads to a degree of control over the absorbing capacity of the powder and the nature of the gel formed on absorbance, due to the variation in the amount of S-sulfo groups present within each protein fraction.

Due to the intact nature of the proteins, and the water solubility of the material arising from the presence of the polar S-sulfonate group, S-sulfonated keratin protein fractions, in particular the keratin intermediate filament protein fraction, can be readily formed into a variety of matrices and the physical properties of these matrices are such that they can provide a useful physical role in a wound environment. Furthermore, the materials can be chemically treated following reformation into films, fibres or sponges, to remove the S-sulfonate functionality and generate disulfide crosslinks within the material, similar to those present in the native keratin. Methods for this treatment are described in NZ/PCT/00169. When treated in this way, the keratin matrices are less absorbing and retain their structure in a wound environment. They are well suited to the delivery of bioactives to the wound site, such as antibacterial agents, growth factors, antibiotic treatments, cultured cells or other drugs.

The biocompatibility for the materials described above has been determined through in vitro growth of cells relevant in wound healing, and immunogenic response, specifically fibroblasts and lymphocytes.

Keratin material	Cystine functionality	Label
Sponge	S-sulfonate	A
Sponge	disulfide	B
Film	S-sulfonate	C
Film	disfulfide	D
Powder	S-sulfonate	E

Table 3: keratin materials tested in vitro.

Sheep Fibroblasts:

Figure 1 is a graph showing the effect of different keratin matrices on ovine dermal fibroblast cell proliferation relative to cell media alone (control). Parallel samples of $n = 3$ were used for each time point.

The proliferation kinetics of ovine fibroblasts on most of the keratin matrices is similar. Wells are initially seeded with ~10,000 cells (0 hours). During the first 24h post-seeding, the culture experiences a lag time as evidenced by the decline in cell numbers. This phenomenon has been recognised in all assays performed and the drop is observed in

control wells in addition to those containing the test materials. Additional shorter time-course experimentation has shown that this lag time lasts for less than 12h (data not shown) and that the exponential phase of growth begins at this point. Population doublings occur approximately every 24h-48h with subconfluency (approximately 80% confluency) marking the end of logarithmic growth. This corresponds to the end of the experimental time course (5 days or 120h). Extended time-course experiments have indicated a plateau in cell growth shortly after this with full confluence of the culture. Contact inhibition and depletion of nutrients play a key role in limiting the growth rate at this point and the monolayer culture exhibits signs of cell death (i.e. loss of membrane integrity, reduction in cell numbers, vacuolisation of individual cells).

Such kinetics are exhibited by sheep fibroblasts on most of the biopolymer substrates, particularly the films and disk samples. Of note, all biopolymer substrates showed lower proliferation rates over the experimental time course compared to those of the control. Statistical testing, using the Student's t test, revealed that these differences were significant (P <0.001).

With respect to the individual matrix types, the following observations were made:

Films. The films with the chemical configuration WSSW support sheep fibroblast growth most satisfactorily (material D). A second configuration ($WSSO_3Na$) demonstrated by film material C supports cell growth to a lesser degree and tends to swell in culture – a trait that may have biofunctional implications for its potential application. Cells on the these films showed typical multi- or bipolar elongated fibroblastic morphology with good spread. Any morphological abnormalities compared to the control were not noted.

Sponge. Fibroblast growth porous sponge material B (WSSW configuration) matched that of some of the better films. During the assay, cells were witnessed to attach to the upper surface of the sponge. By light microscopy, the morphological appearance of these cells was deemed similar on all substrates compared to the no-matrix control. Cells were observed by microscopy to infiltrate the sponge material 7. Proliferation values however do not suggest the sponge to be a good substrate. At time 24h no reading was possible for the sponge cell number as the fluorescent reading obtained was below the limit attainable by the DNA-based assay. Levels rose to recordable levels subsequently but were consistently low. Proposed rationale for this observation was that either the fluorescent dye or DNA bound to the sponge itself, preventing accurate readings. Wax

embedding and sectioning failed to provide answers – it would appear cells were washed off during the dehydration steps.

Powder. A keratin powder dilution series was established and the result presented in the graph as material E. This result represents the observed growth curve for the concentration $2mgml^{-1}$. Higher concentration solutions than this resulted in the

same curve, lower demonstrated a slightly higher cell proliferation rate than the control. Extract tests suggest the keratin powder itself may be, at sufficient concentration, mitogenic for sheep fibroblasts.

Human Fibroblasts:

Figure 2 is a graph showing the effect of different keratin matrices on human dermal fibroblast cell proliferation relative to cell media alone (control)

Human fibroblastic data for the corresponding keratin substrates more or less mirrored that observed with the sheep cell line. Again a typical growth curve was established over the 120h period, however 100% confluence was reached in the control wells by the end of this time. At 120h, cultures grown in the presence of the majority of test materials ranged from 83-89% confluence.

Sheep Lymphocytes

Figure 3 demonstrates the effect of conA stimulation on T cells grown in the presence/absence of keratin matrices over a 10-day period. Tritiated thymidine counts were converted to cell numbers per well (against a series of standards) for each of the treatment groups.

Resulting analysis suggests:

1. There is a marked difference in cell numbers over the 240h experiment between ConA stimulated and non-activated sheep T lymphocyte cells. Control (grown in the absence of keratin biopolymers) cell numbers show a 6-fold difference between unstimulated and stimulated cells at 240h. Cells grown in the absence of conA reached concentrations of 50000 cells/well at Day 10, whilst control cells with ConA supplementation exceeded 300000 cells/well at the same point. Such high

concentrations were obtainable as the cells were maintained in suspension culture therefore reduced nutrient supply and not surface area requirement was the limiting factor.

2. There was little difference in cell proliferation rates between sample (matrix presence) and control (matrix absence) wells. This effect was noted for both stimulated cells and unstimulated cells. In other words:

   (a) Unstimulated cells grown in the presence of matrices proliferated at the same degree as those grown purely on tissue culture reference wells. This indicates that although the keratin biomaterials are not non-immunogenic, they look to be antigenically inert. If they were non-immunogenic, one would expect no proliferation of lymphocytes exposed to the biomaterial. If indeed it is inert, cell proliferation rates would mimic those of the control as appears the case.

   (b) Stimulated cells grown in the presence of the matrices proliferated at a similar rate or slightly higher than the control wells (which contained no keratin matrix). This suggests the activated T-cells are not being inhibited in any way by the matrix itself or any degradative by-products it may produce over this short time-course. Failure to inhibit active T-cells by the tested biomaterials demonstrates the product does not interfere with the normal cell-mediated immune response.

Figure 4 shows the effect of ConA stimulation on T-lymphocytes cells grown in the presence of a variety of matrices at 72h. Total counts reflect the level of thymidine uptake and incorporation into DNA, which is then used as a measure of proliferation (see in the previous graph). A 72h culture is regarded as the best measure of time for comparison between treatments as the cells are well within exponential phase growth.

The results are presented as a vertical bar graph with stimulated and non-stimulated treatments beside each other. Error bars represent means ± SD for $n = 3$. The unstimulated well counts (unlabelled) show very little variation with small error scores. Total counts for stimulated cells are slightly more variable although student T test analyses indicate only the material C is significantly different ($p = 0.075$) from the control.

Conclusions

Unprimed T cells were shown to proliferate at the same degree in the presence or absence of the matrices. This showed the biomaterials were not non-immunogenic but instead inert. No single matrix tested stimulated the normal immune response to any degree greater than the control (no matrix well series).

Activated T cells maintained in culture with keratin matrices proliferated normally at a similar or greater than rate compared to those of the control (no matrix present). This demonstrates the biomaterials are biocompatible with stimulated T cells, mitogenic to a degree and most certainly do not interfere with the normal immune response. There was no inhibition of activated T cells by any matrix or its byproducts.

In conclusion, the tested matrices do not interfere with the body's cell-mediated immune response and are biocompatible with a sheep T lymphocyte cell line.

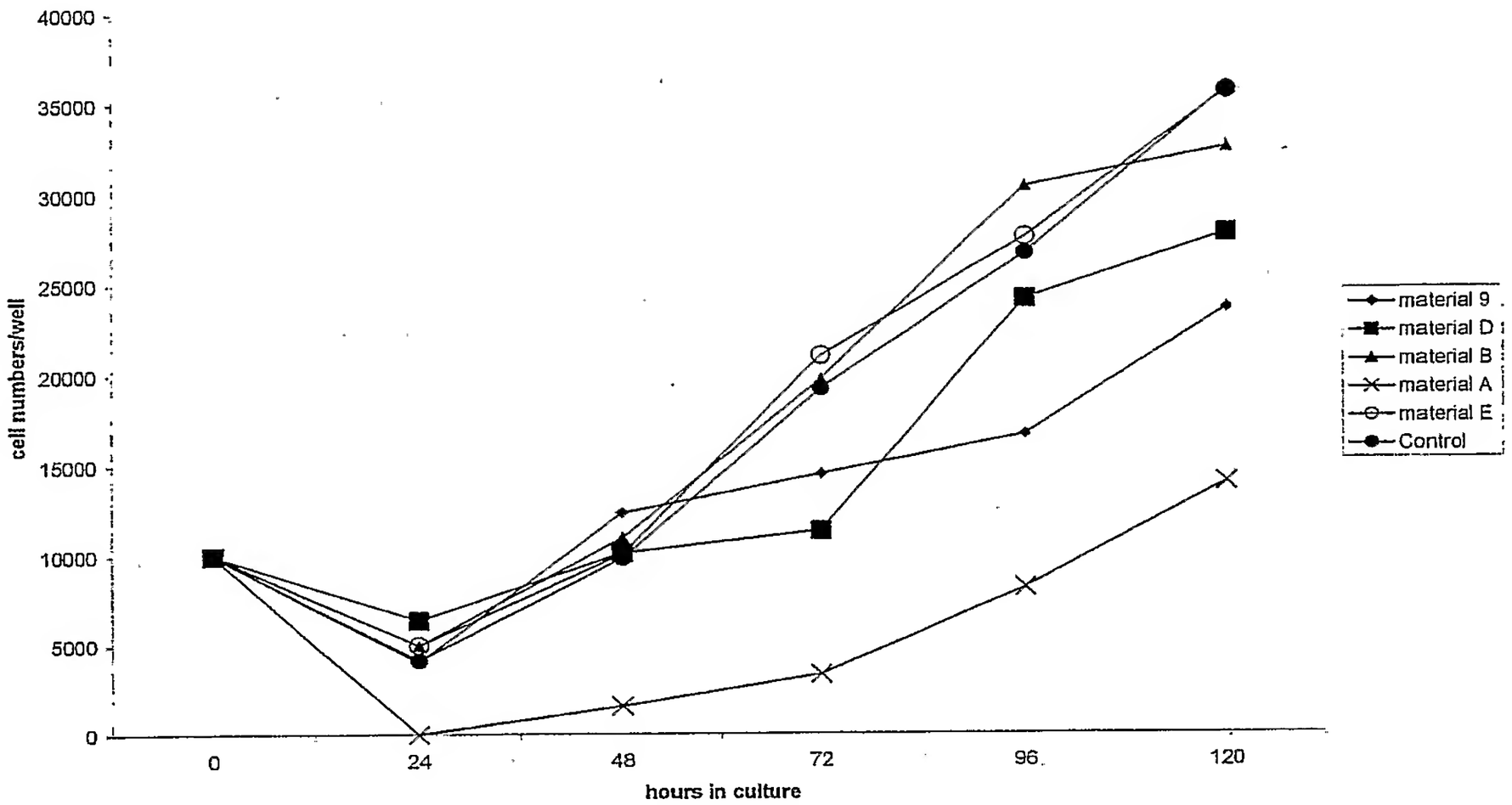
Whilst the invention has been described with reference to particular embodiments, it will be appreciated that numerous modifications and improvements may be made to the embodiments without departing from the scope of the invention as described in this specification.

WOOL RESEARCH ORGANISATION OF NEW ZEALAND (INC.)

By their Attorneys

BALDWIN SHELSTON WATERS

Figure 1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15000
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48
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96
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material D
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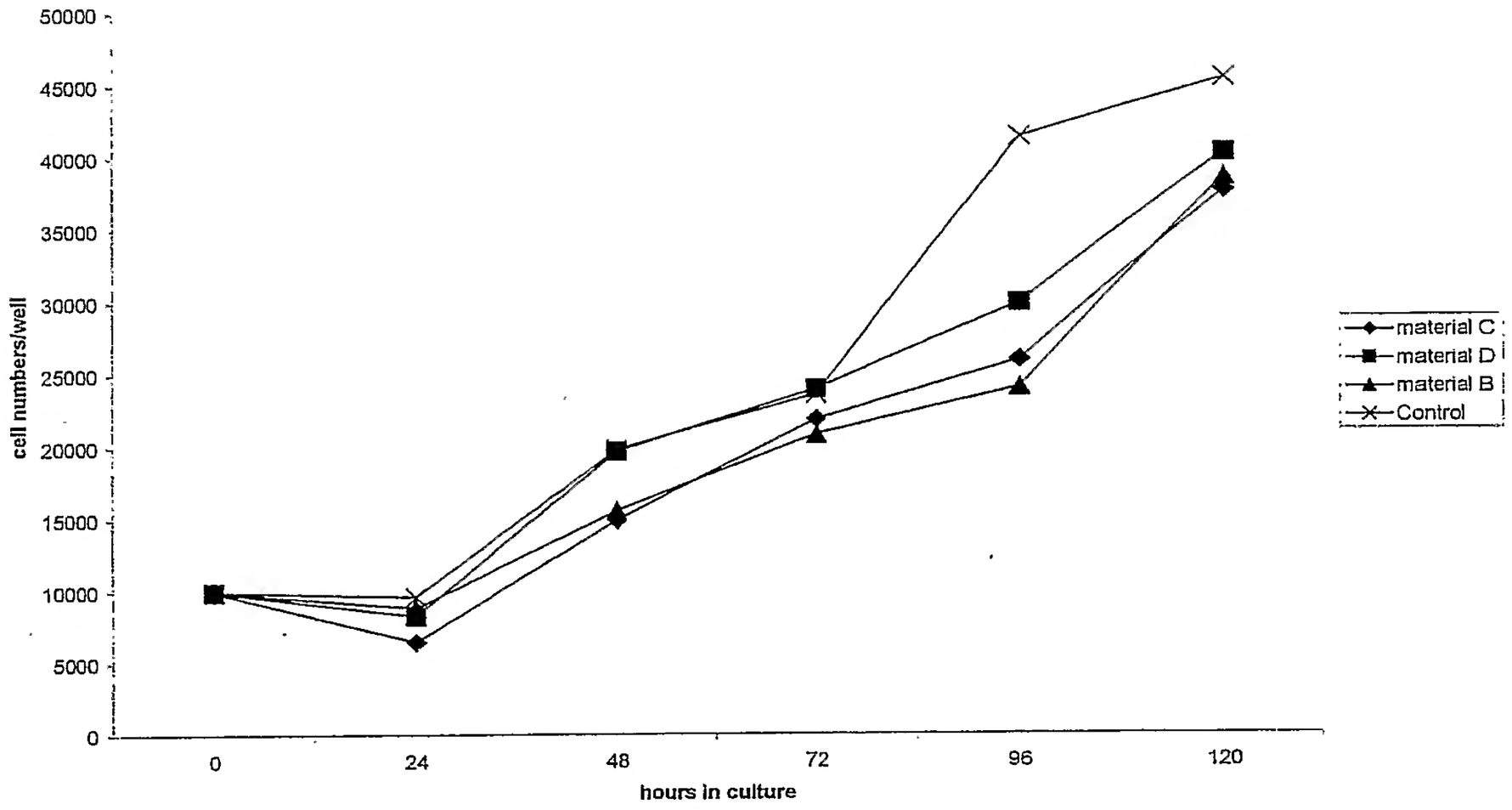
material E
Control

Figure 2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20000
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48
72
96
120
hours in culture
material C
material D
material B
Control

Figure 3

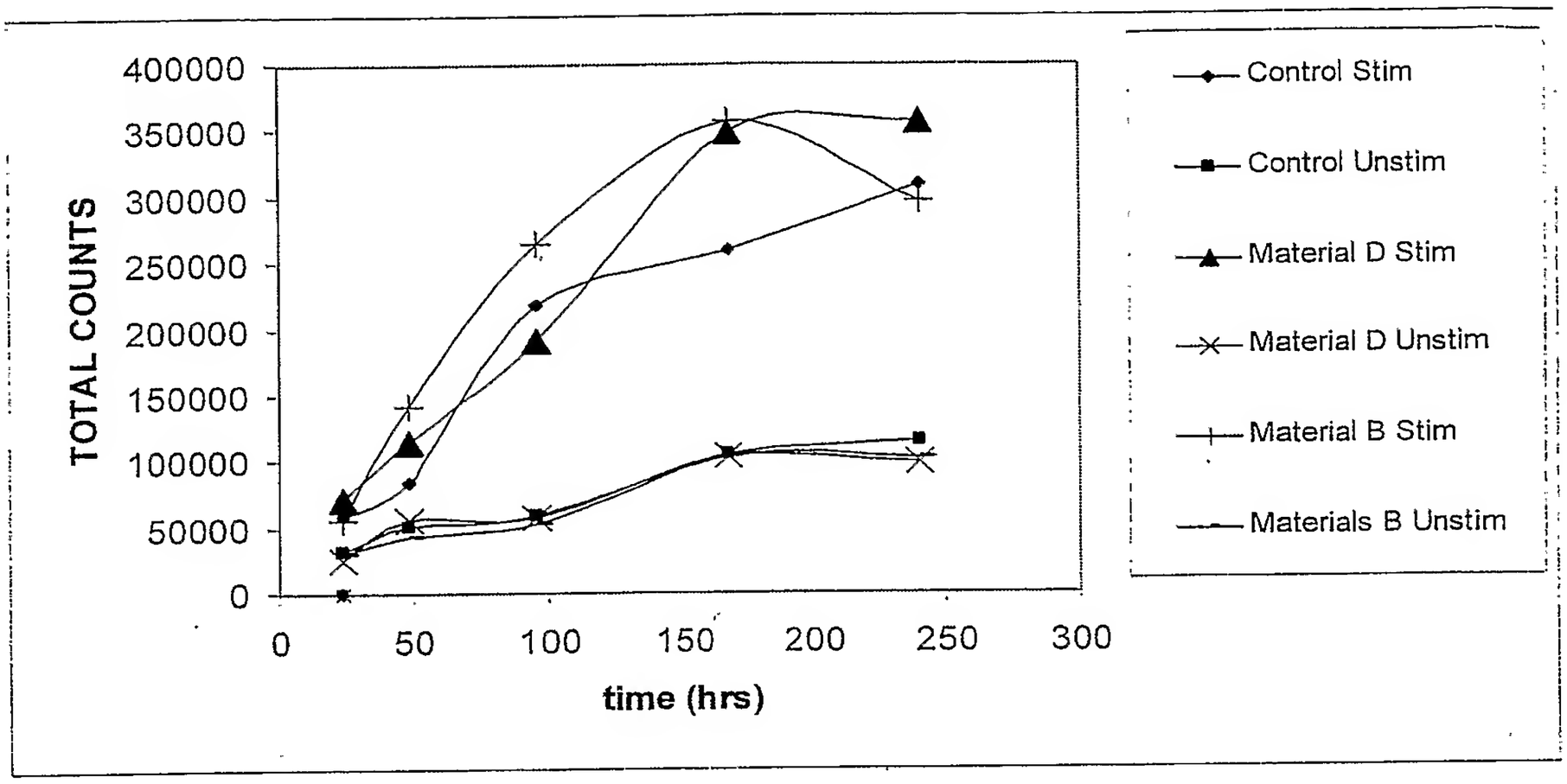

Figure 4